Cartilage tissue engineering using adipose stem cells

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RAPID ATTACHMENT OF ADIPOSE STROMAL CELLS ON RESORBABLE POLYMERIC SCAFFOLDS FACILITATES THE ONE-STEP SURGICAL PROCEDURE FOR CARTILAGE AND BONE TISSUE ENGINEERING PURPOSES

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ABSTRACT

The stromal vascular fraction (SVF) of adipose tissue provides an abundant source of mesenchymal stem cells. For clinical application, it would be beneficial to establish treatments in which SVF is obtained, seeded onto a scaffold, and returned into the patient within a single surgical procedure. In this study, we evaluated the suitability of both a macroporous poly(L-lactide-co-caprolactone) and a porous collagen type I/III scaffold for this purpose. Surprisingly, cell attachment was rapid (~10 min) and sequestered the majority of adipose stem cells, as deduced from colony-forming unit assays. Proliferation occurred in both polymeric scaffolds. Upon chondrogenic induction, up-regulation of chondrogenic genes, production of glycosaminoglycans, and accumulation of collagen type II was observed, indicating differentiation of scaffold-attached SVF cells along the chondrogenic lineage. Osteogenic differentiation was achieved in both scaffold types, as visualized by up-regulation of osteogenic genes, increase of alkaline phosphatase production over time, and accumulation of bone sialoprotein and osteonectin. In conclusion, this study identifies both poly(L-lactide-co-caprolactone) and collagen type I/III as promising scaffold materials for rapid attachment of adipose stem cell-like (stromal) cells, enhancing the development of one-step surgical concepts for cartilage and bone tissue engineering.

INTRODUCTION

Adipose tissue provides an abundant source of multi-potent adipose tissue-derived stem cells (ASCs) which have the capacity to differentiate into adipocytes, chondrocytes, osteoblasts, myocytes, cardiomyocytes, and hepatocytes in vitro.[1] In vivo, the osteogenic differentiation potential of ASCs has been used to heal critical-size skeletal defects in mice[2] and widespread calvarial defects in a child,[3] whereas the chondrogenic differentiation capacity has been demonstrated in mice and rabbits.[4,5]

Because it is possible to obtain clinically relevant numbers of ASCs with minimally invasive techniques,[6] our group recently proposed a so-called one-step surgical procedure for bone and cartilage regeneration using ASCs.[7] Briefly, the surgeon harvests adipose tissue which is subsequently processed to obtain the stromal vascular fraction (SVF) containing the ASCs. Without culture expansion, the SVF is then seeded onto a scaffold material. Finally, the ASC-scaffold construct is implanted, all in the same surgical procedure. Obvious advantages of this approach in humans would be not only its patient-friendliness but also its lower costs, since a second surgical intervention and expensive in vitro culturing steps would be avoided.

Since stem cells are one of the corner stones of tissue engineering, the feasibility of a one-step procedure depends on the ability of the ASC fraction within the SVF to attach to a scaffold material in sufficient quantities, but moreover within a short time frame, that is, within a few hours or less. Furthermore, the attached cells should be able to differentiate along the chondrogenic and osteogenic lineage in order to enhance the clinical outcome.

In this study, we evaluated two polymeric scaffolds: a synthetic biodegradable 70:30 poly(L-lactide-*co*-caprolactone)(PLCL) scaffold and a natural collagen type I/III (Optimaix®) scaffold (Col I/III) with directed pores. The aim of this in vitro study was to establish whether non-cultured SVF cells could be used in the framework of a one-step surgical procedure. We focused on (i) kinetics of SVF attachment and proliferation on both scaffold materials, and (ii) differentiation of attached SVF cells along the chondrogenic and osteogenic lineage.

MATERIALS AND METHODS

Tissue Sampling and Processing

Human subcutaneous adipose tissue samples were obtained as waste material after elective surgery upon informed consent from patients of the VU University Medical Center. SVF isolation was performed as described previously.[8] Briefly, resection material was first cut into small pieces and digested enzymatically with 0.1% collagenase A (Roche Diagnostics GmbH, Mannheim, Germany). Single cells were obtained by passing the digest over a 200 mM mesh filter. Subsequently, cells were subjected to a ficoll density centrifugation step to remove erythrocytes.

Scaffolds

The 70:30 poly(L-lactide-*co*-caprolactone) scaffolds (kindly provided by Cytori Therapeutics, San Diego, CA) had isotropic and interconnected pore sizes between 250–425 mm and

a porosity of 89%. Before use, scaffolds were rinsed with 70% ethanol, washed with phosphate-buffered saline (PBS) and incubated for 1 h in serum containing medium (DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine, further referred to as culture medium) in a humidified incubator, using a procedure developed by Ishaug-Riley et al.[9]

The collagen type I/III scaffold (Optimaix®, Matricel GmbH, Herzogenrath, Germany) had a porosity of 98%, with pore sizes around 85 mm.[10] Cells were seeded directly onto the scaffold material, according to the manufacturer's drop-on protocol.

Kinetics of Cell Adhesion to Scaffolds

Freshly isolated SVF cells (further referred to as SVF) of eight donors (age 39.8 ± 9.7 years, BMI 25.5 ± 2.4, 397,498 ± 180,381 nucleated cells/gram adipose tissue) were compared for attachment to both scaffolds (6 mm diameter and 1.5 mm high). Fifty thousand SVF cells were seeded per scaffold and allowed to attach in a humidified incubator (37°C, 5% CO_2) for 5–120 min. Prior to analysis, scaffolds were washed with PBS. The number of attached cells was quantified using a CyQUANT® Cell Proliferation Assay Kit (Invitrogen, Breda, The Netherlands).

Colony-Forming Unit Fibroblast (CFU-F) Assays

To assess the ASC frequency in SVF and the frequency of these ASCs capable of attaching to the scaffold materials, CFU-f and CFU-f depletion assays were performed (n = 7). Frequencies were expressed as a percentage by dividing the number of colonies by the number of cells seeded.

For the CFU-f depletion assays, SVF cells were first seeded as described above for 60 min (n = 7). Non-attached cells, obtained by washing the scaffolds with PBS were collected, and tested for CFU-f frequencies at 10-fold higher densities as "normal" CFU-f since it was established empirically that seeding at the "normal" density resulted in indistinct colonies.

Both the primary and the depletion CFU-f assay cells were grown for 14–21 days, fixed in 4% paraformaldehyde, and stained with a 0.2% toluidin blue solution in borax buffer for 1 min.

Proliferation of SVF Cells in the Scaffold

Proliferation of SVF cells in the scaffolds was assessed by seeding 50,000 SVF cells (see Kinetics of Cell Adhesion to Scaffolds Section; 60 min attachment), followed by proliferation in culture medium for up to 14 days. Cell quantities were determined using the CyQUANT® Kit (Invitrogen) as described above.

Chondrogenic and Osteogenic Differentiation of Attached SVF Cells in the Scaffolds

Chondrogenic differentiation was induced by dropping a 30 µl cell suspension containing 2 x 10^6 SVF cells onto the scaffolds (attachment for 60 min). Then, 1 ml of chondrogenic medium (culture medium supplemented with 1% ITS+ ™ Premix (BD Biosciences, Breda, The Netherlands), 10 ng/nl transforming growth factor-β1 (TGF-β1, ITK-diagnostics, Uithoorn, The Netherlands), 1% FCS (Hyclone), and 25 mM ascorbate-2-phosphate

(Sigma-Aldrich, Zwijndrecht, The Netherlands) was overlaid gently. Cell-loaded constructs were cultured up to 21 days and medium was changed every other day.

For osteogenic differentiation, 2 x 10^5 SVF cells were seeded per scaffold. SVF cells were cultured in osteogenic medium consisting of culture medium supplemented with 10 mM β-glycerol phosphate, 50 µg/ml ascorbate-2-phosphate, and 100 ng/ml bone morphogenetic protein 2 (BMP-2, Peprotech EC LTD, London, UK). Cell-loaded constructs were cultured up to 14 days and media were changed twice a week.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA isolation was performed using the Magnapure[1] system and the Magnapure® LC RNA isolation kit III (Roche Diagnostics GmbH). cDNA synthesis was performed using Masterscript Vilo®. Real-time PCR reactions were performed using the SYBRGreen reaction kit in a LightCycler 480 (Roche Diagnostics GmbH). cDNA (approximately 5 ng) was used in 20 ml PCR mix (LightCycler DNA Master Fast start [plus] Kit, Roche Diagnostics GmbH) containing a final concentration of 0.5 pmol of primers. Relative housekeeping gene expression (YWHAZ and HPRT), and relative target gene expression (chondrogenic differentiation: aggrecan (AGG), collagen types IIa and X, cartilage oligomeric matrix protein (COMP), link protein (LINK) and the early transcription factors L-SOX-5, SOX-6, and SOX-9; osteogenic differentiation: alkaline phosphatase (ALP), collagen type I, Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and osteonectin (SPARC)) were determined using previously described primer sets.[11,12]

With Light Cycler software version 4, crossing points were assessed, and plotted versus a serial dilution of individual gene standards using Fit Points method. PCR efficiency was calculated, and only data with efficiencies ranging from 1.85 to 2.0 were used.

Immunofluorescent Staining of Chondrogenic and Osteogenic Matrix Formation

Immunohistochemistry was performed on cell-loaded scaffolds fixed in 4% paraformaldehyde for 30 min. Cell-loaded scaffolds cultured under chondrogenic conditions were stained for collagen type II (mouse-anti-human monoclonal antibody II-II6B3 [1:50; Developmental Studies Hybridoma Bank]) in PBS/1% bovine serum albumin (BSA)/0.1% Saponin (Sigma-Aldrich) overnight. Cell loaded constructs induced towards the osteogenic lineage for 14 and 21 days were, after pre-incubation for 10 min in normal goat serum (1:20), immunostained with 1:200 dilutions of either a rabbit-anti-human osteonectin (SPARC) antibody (EMD Biosciences, Inc., La Jolla, CA) or a rabbit-antihuman bone sialoprotein (BSP) antibody (Immundiagnostik, Bensheim, Germany) overnight at 4°C (negative controls: non-immune serum).

Fluorescent staining of all antigens was performed by incubation for 1 h with a 1:400 dilution of Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen). Cell nuclei were visualized by staining with 7.5 mM propidium iodide (Sigma-Aldrich) for 15 min. Human trachea and mandibular sections served as positive chondrogenic and osteogenic controls and non-induced cell-loaded constructs as negative controls (incubated with rabbit IgG1), respectively (data not shown).

Immunostained cells within the scaffolds were detected using confocal microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany). Top view projections were made

by taking a *z*-stack (50 images) of the scaffold, using the maximum projection algorithm of the Leica software. Detector gains, laser powers, averaging modes, etc. were first set to values at which no expression was detected in negative controls, which were subsequently applied for all samples.

Extracellular Matrix Biosynthesis

Deposition of extracellular sulfated glycosaminoglycans (sGAG) in chondrogenically induced cell-loaded scaffolds was quantified in 14-and 21-day samples as previously described.[12] In short, tissue within scaffolds was digested in 3% papain solution buffer overnight at 60ºC. sGAG were measured using a Blyscan kit (Biocolor Ltd, Carrickfergus, Ireland).

ALP activity and DNA content were quantified in parallel in cell lysates of cell-loaded scaffolds after 4 and 21 days of osteogenic induction, as previously described.[11,13] Briefly, the substrate P-nitrophenyl phosphate (Merck, Darmstadt, Germany) at pH 10.3 was used to determine cellular ALP activity, whereas DNA content was assessed with the CyQUANT® Cell Proliferation Assay Kit as described above.

Statistics

All experiments were performed using cells of 4–8 different donors. Kolmogorov–Smirnov tests were used to determine normalcy of measurements and, if appropriate, their logarithmics. For evaluation of CFU-f assays and proliferation, means between two groups in one variable were compared using the Student's t-test. For evaluation of attachment, repeated measures ANOVA was used. For evaluation of induction of specific genes, a non-parametric Mann–Whitney test was performed. Gene expression levels below the detection limit (0.05) were set at 10^2. All statistical tests used a significance level of $\alpha = 0.05$.

RESULTS

Kinetics of SVF Cell Attachment

SVF cells already attached to both 70:30 poly(L-lactide-co-caprolactone) scaffolds and collagen type I/III scaffolds within 5-10 min after cell seeding. The percentage of attached cells increased to about 10.2 ± 2.6% (mean ± SEM) after 30 min for the PLCL scaffold, whereas 4.5 ± 1.4% attached to the collagen scaffold (Fig. 1a), and stabilized thereafter. Overall, significantly more cells attached to the PLCL scaffold compared with the collagen scaffold ($p < 0.01$).

To assess whether preferentially stem cell-like cells attached to the scaffolds, pre-and post-seeding (depletion) CFU-f assays were performed. To correct for donor variation, a treatment over control ratio (T/ C) was applied by dividing the percentage of the depletion CFU-f by its corresponding percentage of pre-seeding CFU-f. Figure 1b shows a significant decrease in the T/C ratio for both the PLCL (0.19 ± 0.15) and Col I/III (0.12 ± 0.09) scaffolds indicating that for both scaffolds, the non-attached cell-fraction contained significantly less ASCs ($p < 0.001$).

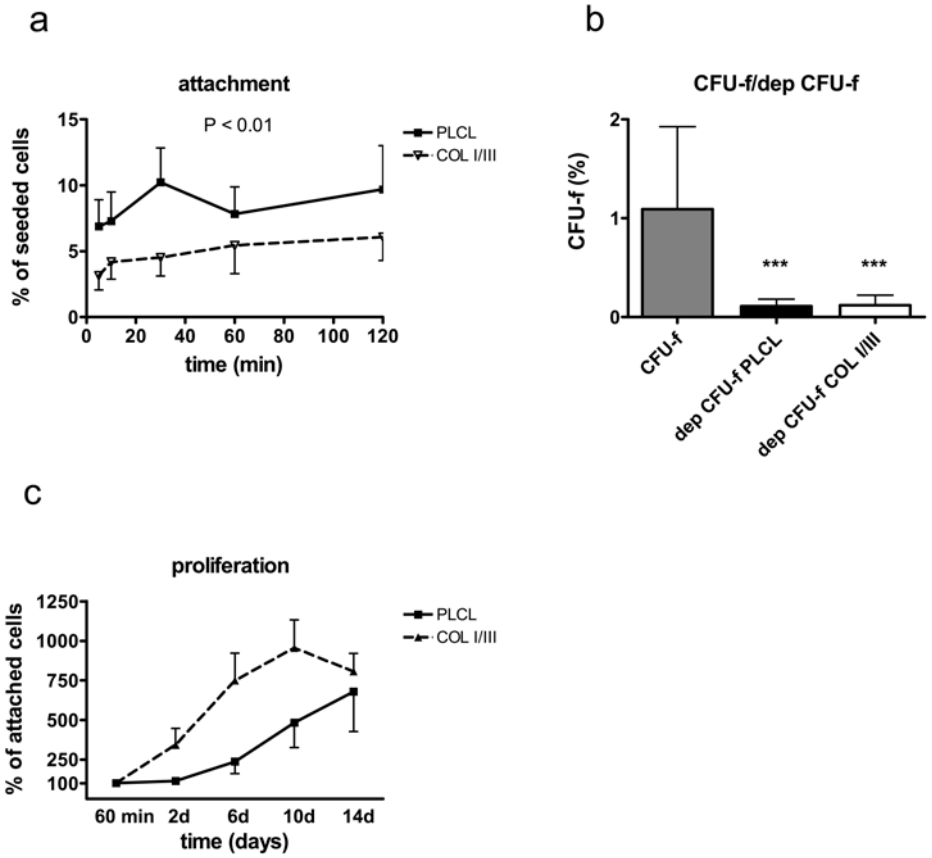

Figure 1. *a: Frequency of freshly isolated SVF cells attached to 70:30 poly(L-lactide-co-caprolactone) scaffolds (PLCL) and collagen type I/III (COL I/III) scaffolds, expressed as % of the number of seeded cells. A significant difference was found between PLCL and COL I/III, p < 0.01. b: Cell proliferation in PLCL and COL I/III scaffolds. The SVF cell numbers expressed as % of the number of cells that attached after 1 h (set at 100%) are depicted. c: Preseeding and post-seeding (depletion) CFU-f assays. A treatment over control ratio (T/C) was applied to the CFU-f assays (depletion CFU-f/preseeding CFU-f). A significant decrease for both scaffolds in CFU-f ratios can be seen, compared to the preseeding CFU-f (p < 0.001).*

Proliferation of SVF Cells in the Scaffold

Cell numbers within scaffolds increased from days 2 to 10 and stabilized thereafter, culminating to more than eightfold and ninefold increases at day 14 for PLCL and collagen scaffolds, respectively (Fig. 1c). Although not significant, a clear trend was visible towards higher proliferation rates in the Col I/III scaffold. Mean population doubling times (n = 5) were 2.4 ± 0.3 days (mean ± SD) for the PLCL and 1.6 ± 0.8 days (mean ± SD) for the Col I/III scaffold, similar to proliferation rates previously reported for ASCs cultured on tissue culture-treated plastic.[9]

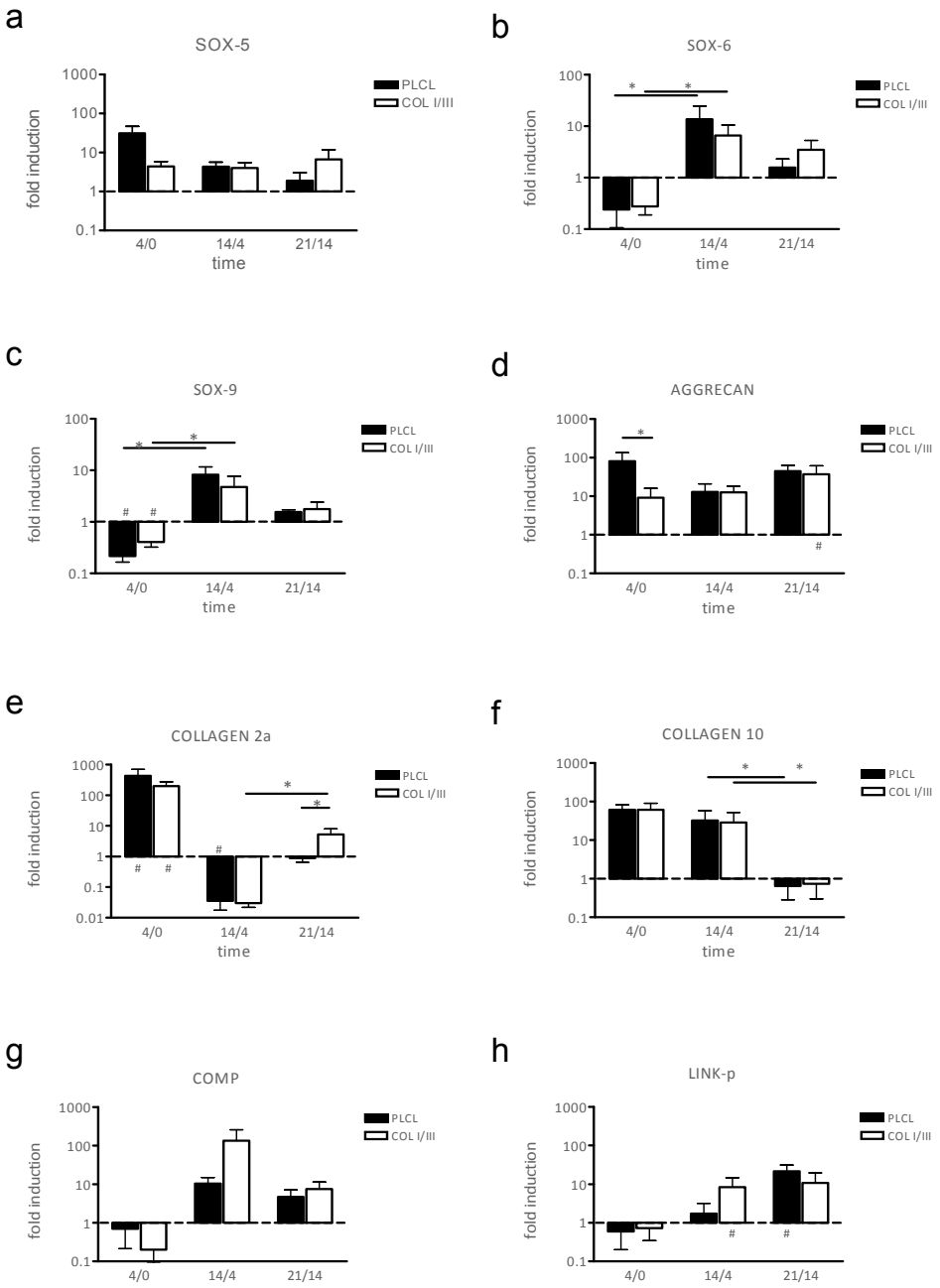

Figure 2. *Chondrogenic gene induction of SVF cells seeded onto PLCL and COLI/III scaffolds at increasing time-points (days 0, 4, 14, 21). a–h: Down- and up-regulation of chondro-specific genes. Asterisks represent differences in kinetics of gene up-regulation. # Significant difference in gene induction compared to the previous time-point. COMP, cartilage oligomeric matrix protein; LINK-p, link-protein; SOX 5,6,9, Sry-related HMG box 5,6,9.*

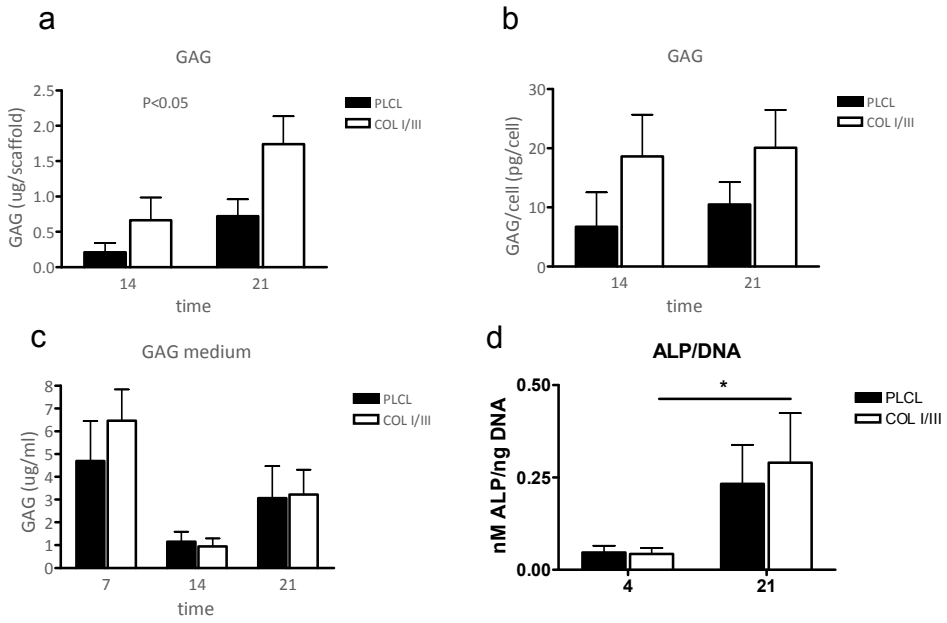

Figure 3. *Confocal images of SVF cells seeded onto PLCL and COL I/III scaffolds, cultured in chondrogenic medium for 21 days. Cell nuclei are stained in red using propidium iodide. Green staining indicates positivity for collagen type II. Collagen type II is clearly visible in both the PLCL scaffold (a) and the COL I/III scaffold (b). c,d: Magnification of a and b showing intra- and extracellular collagen type II deposition.*

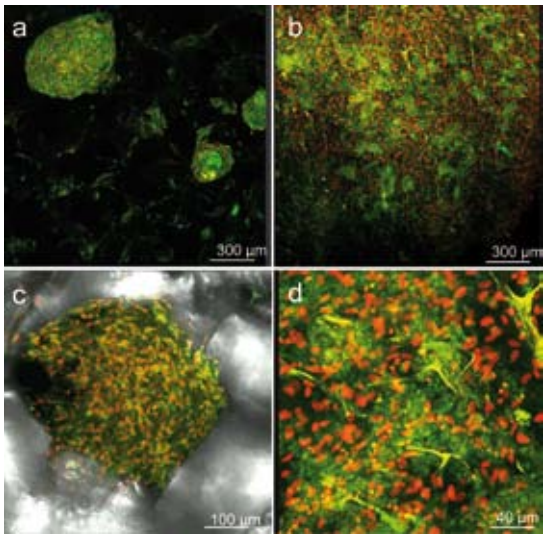

Figure 4. *Quantification of chondrogenic matrix deposition (glycosaminoglycan (GAG) analysis) and osteogenic differentiation (alkaline phosphatase (ALP) activity) in PLCL and COLI/III scaffolds. a:GAG increase over time in both scaffold types. In COLI/III, significantly more GAG was produced compared to PLCL. b: GAG synthesis corrected for the total number of cells in the scaffold (c) GAG secretion in the medium. The decreased GAG production after 14 days is likely due to sequestering of GAG in the extracellular matrix. d: ALP activity in osteogenically induced scaffolds. A trend in increased ALP activity over time for the PLCL scaffold and a significant increase over time for the COLI/III scaffold ($p < 0.05$) are observed.*

Chondrogenic Differentiation of SVF Cells in the Scaffold

Upon chondrogenic induction of SVF cells in both scaffolds for up to 21 days, RNA expression was determined at several time-points using quantitative RT-PCR. The early transcription factors SOX-9 and SOX-6, as well as the late genes COMP and LINK were down-regulated between 0 and 4 days (all $p < 0.05$, Fig. 2b,c,g,h) compared to non-induced controls, but increased in the 4–14 days, and even stronger in the 14–21 days interval of induction in both scaffold types. The early gene COL2A was up-regulated after 4 days in both scaffolds (Fig. 2e, $p < 0.05$), and down-regulated after 14 days of induction. After 21 days it was down-regulated further in the PLCL and up-regulated in the collagen scaffold ($p < 0.05$). SOX-5 (Fig. 2a) and AGG (Fig. 2d) gene expression were up-regulated at all time points and after 4 days a significant difference was found in AGG gene up-regulation when comparing both scaffolds ($p = 0.035$). The hypertrophic marker COLX was up-regulated up to 14 days of induction in both scaffolds, followed by down-regulation at 21 days ($p < 0.05$, Fig. 2f).

Collagen type II immunostaining confirmed chondrogenic extracellular matrix deposition: constructs stained slightly positive at 14 days, and staining intensity increased after 21 days of induction in both scaffolds (Fig. 3). Detailed analysis (Fig. 3c,d) shows positive staining for collagen type II in both the cellular cytoplasm and the extracellular matrix of both constructs.

Biochemical analysis showed a temporal increase in GAG formation in both materials, with a significant difference ($p < 0.05$) between the PLCL and COL I/III scaffolds (Fig. 4a). Upon correction for cell numbers, still a clear trend in the GAG production could be seen between both scaffold materials (Fig. 4b). GAG in the medium was found to be highest after 7 days, decreased after 14 days, and increased again after 21 days of induction, although these differences were not statistically significant (Fig. 4c).

Osteogenic Differentiation of SVF Cells in the Scaffold

Upon osteogenic induction of SVF cells in both scaffolds for up to 21 days, RNA expression was determined after increasing time-points (0, 4, 14 days) using quantitative RT-PCR (Fig. 5). At 4 days all osteogenic genes were down-regulated compared to non-induced controls, with values reaching significance only for the Col I/III scaffold for RUNX2 ($p < 0.05$, Fig. 5a), ALP ($p < 0.05$, Fig. 5b), and SPARC ($p < 0.01$, Fig. 5d). For the 14-day time-point, trends in up-regulation could be seen for RUNX2, OCN (Fig. 5c) and, with respect to the PLCL scaffold, for COL1a. Significant up-regulation was seen in the Col I/III scaffold for COL1a, ALP and SPARC gene expression ($p < 0.05$, Fig. 5e,b,d). PLCL scaffolds showed up-regulation after 14 days in ALP ($p < 0.05$) and SPARC expression ($p < 0.01$). No significant differences could be detected for all time points when comparing the data from both scaffolds. Gene expression over time showed significant up-regulations for the COL1a and SPARC genes in both scaffolds (Fig. 5d,e).

Immunohistochemical analysis of osteogenic extracellular matrix deposition showed no staining at 7 days (data not shown), but a clear immunostaining for SPARC (Fig. 6a,c) and BSP (Fig. 6b,d) proteins after 21 days. In support, biochemical analysis of the osteogenically induced constructs (Fig. 4d) showed an increasing trend in ALP activity over time for the PLCL and a significant increase for the COL I/III scaffold ($p < 0.05$). No significant differences were observed in ALP activity between both scaffold materials.

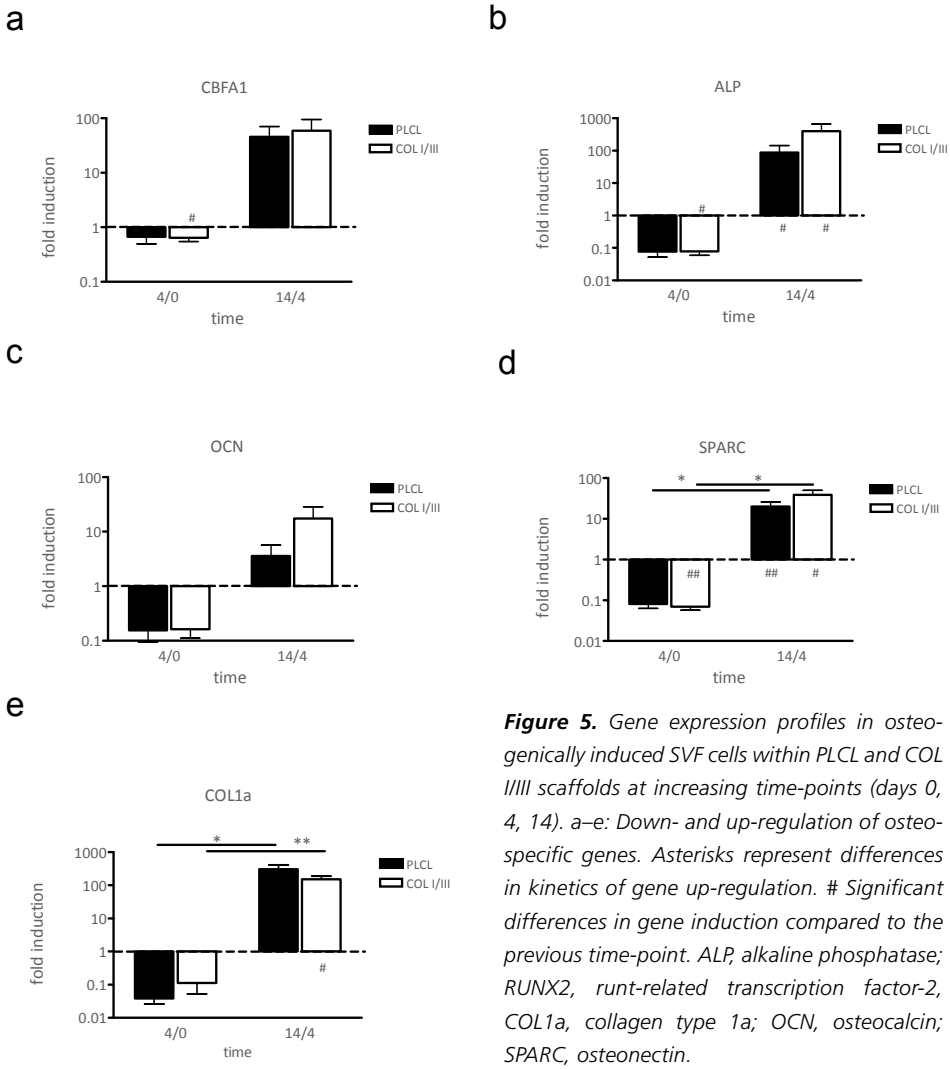

Figure 5. *Gene expression profiles in osteogenically induced SVF cells within PLCL and COL I/III scaffolds at increasing time-points (days 0, 4, 14). a–e: Down- and up-regulation of osteo-specific genes. Asterisks represent differences in kinetics of gene up-regulation. # Significant differences in gene induction compared to the previous time-point. ALP, alkaline phosphatase; RUNX2, runt-related transcription factor-2, COL1a, collagen type 1a; OCN, osteocalcin; SPARC, osteonectin.*

DISCUSSION

In this study, we tested two radiolucent biodegradable polymeric scaffolds, a natural porous COL I/III scaffold and a macroporous poly(L-lactide-co-caprolactone) scaffold for applicability within a one-step surgical procedure (OSP). This OSP employs intraoperatively isolated adipose tissue-derived stromal vascular fractions (SVF), which contain clinically relevant amounts of adipose mesenchymal stem cells (ASCs). We evaluated the scaffolds with particular emphasis on the attachment, proliferation and differentiation profiles of these ASCs. From the data presented in this study we conclude that (i) SVF attachment to both scaffold materials was very rapid (~10 min.), (ii) mainly ASC-like cells attached to both

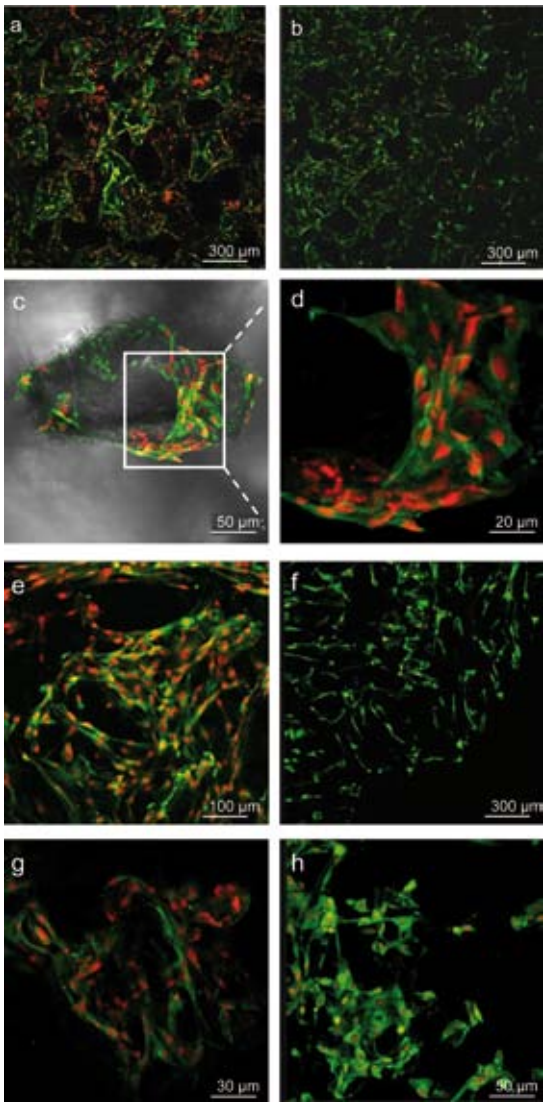

Figure 6. *Confocal images of SVF cells seeded onto PLCL and COL I/III scaffolds cultured in osteogenic medium. Red: propidium idodide stained cell nuclei. Green staining indicates cells stained positive for either osteonectin (a–d) or bone sialoprotein (e–h). (a, b) Expression of osteonectin after 21 days in PLCL and COL I/III scaffolds, respectively. (c) Detail of (a). (d) Magnification of (c) in which the scaffold material is not visualized for clarity. (e–h) BSP staining after 21 days in PLCL (e) and COL I/III scaffolds (f). (g, h) Magnification of (e, f) for detailed intraporous deposition of BSP.*

scaffold materials, and (iii) cells in the scaffold were able to proliferate and differentiate towards the chondrogenic and osteogenic lineage.

The rapid attachment to the macroporous 70:30 poly(L-lactide-*co*-caprolactone) and collagen type I/III scaffolds is remarkable, since freshly isolated ASCs normally require approximately 24 h to attach to tissue culture plastic (TCP).[14] Since culturing for >24 h excludes these cells from the Food and Drug Administration (FDA) criteria for minimal manipulation of stem cells,[15] this commonly used adherence selection method hampers feasibility and applicability of stem cell technology in surgical disciplines considerably. Our findings therefore bring one-step surgical procedures closer to clinical practice.

At present, the underlying mechanism for the rapid and selective attachment of the ASC-like subpopulation to these scaffolds is not fully understood. In our experiment cell attachment was presumably mediated by serum proteins adsorbed to the scaffold, since it was shown that mesenchymal stem cells barely attached to various polymeric scaffolds in serum-free conditions.[16] Further studies are warranted to address these issues in more detail, but in this regard it is interesting to note that follow-up experiments showed that omitting the serum precoating step did not noticeably affect the attachment profiles (data not shown).

Cell attachment to the scaffold materials was analyzed by evaluation of DNA using CyQUANT analysis. Although this method is well established and validated for quantitatively analyzing cell attachment and proliferation,[17] it cannot differentiate between different cell types in a heterogeneous mixture of cells like SVF. Therefore we performed colony-forming unit assays and colony-forming unit depletion assays to determine whether the adipose stem cells in the heterogenous SVF attached to the scaffold material. To the best of our knowledge, this is the first time that selective cell attachment was evaluated in this way.

In both scaffold materials SVF cells differentiated into the osteogenic and chondrogenic lineage upon induction with lineage specific medium containing BMP-2 or TGF-β1, respectively. For in vivo applications, we envision either pre-implantation induction strategies during the one-step surgical procedure (feasibility shown by Knippenberg et al.[18]), or induction by the microenvironment after implantation, as suggested by us and others.[19,20] We could not detect any significant differences in the osteogenic differentiation capacity of the SVF on both scaffold materials on both the genetic and the protein level. However, when inducing cells into the chondrogenic lineage, significant differences were detected in both up-regulation of chondrospecific genes as in the deposition of extracellular matrix in favor of the collagen type I/III scaffold. Whether this can be contributed to differences in scaffold architecture or scaffold material properties remains unclear, but similar results have been observed by other authors.[21] Nevertheless, these results underline the importance of scaffold design in conducing or even inducing cells into a specific lineage.

In summary, our experimental findings demonstrating a rapid and selective attachment of freshly isolated adipose derived stem cells to both radiolucent 70:30 poly(L-lactide-*co*-caprolactone) and collagen type I/III scaffolds augment the development of a cost-effective, patient-friendly one-step surgical procedure for cartilage and bone tissue engineering purposes. However, mechanical considerations favor the use of the PLCL scaffold for bone tissue engineering, whereas the flexible collagen type I/III scaffold appears more suitable for engineering of cartilage tissue.

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